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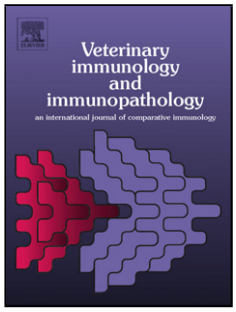

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Synergistic effect of co-stimulation of membrane and endosomal TLRs on chicken innate immune responses

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Highlights

- Macrophages from older birds showed higher immune responses than from young birds.
- Co-stimulation with TLR21 and TLR4 ligands synergistically enhanced immune responses.
- High concentration of CpG B induced cell death, but not cell proliferation.

Abstract

Toll-like receptor (TLR) ligands (TLR-Ls) are critical activators of immunity and are successfully being developed as vaccine adjuvants in both mammals and birds. In this study, we investigated the synergistic effect of co-stimulation of membrane and endosomal TLRs on the innate immune responses using chicken bone marrow-derived macrophages (BMMs), and studied the effect of age on the induction of innate responses. BMMs from 1 and 4-week-old birds were stimulated with Pam3Cys-SK4 (PCSK; TLR2), synthetic monophosphoryl lipid A (MPLA), Di[3-deoxy-D-manno-octulosonyl]-lipid A ammonium salt (KLA; TLR4), Gardiquimod, Resiquimod (R848; TLR7), CpG class B and C (TLR21). Nitric oxide (NO) production and mRNA levels of IL-1β, IL-10 and IL-12p40 showed macrophages from 4-week-old birds showed more sensitive responses compared to 1-week-old birds. The most potent TLR-Ls, PCSK, MPLA and CpG B were used to study the effect of co-stimulation on macrophages. Co-stimulation with TLR21 and TLR4 synergistically up-regulated inflammatory-related genes, as well as NO production. However, incubation of splenocytes with PCSK, MPLA and CpG B did not induce cell proliferation. Moreover, treatment with CpG B led to significant cell death.

Abbreviations

TLR, Toll-like receptor; TLR-Ls, Toll-like receptor ligands; BMMs, bone marrow-derived macrophages; PCSK, Pam3Cys-SK4; MPLA, synthetic monophosphoryl lipid A; KLA, Di[3-deoxy-D-manno-octulosonyl]-lipid A ammonium salt; R848, Resiquimod; CpG, Cytosine-phosphate-guanosine; CpG ODN, CpG oligodeoxynucleotides; CpG B, class B CpG ODN; CpG C, class C CpG ODN; hr, hours; sec, seconds

Key words: Chicken, Toll-like receptor, macrophage, cytokine, adjuvant

1. Introduction

Toll-like receptor ligands (TLR-Ls) are potent immunostimulants with profound effects on the generation of adaptive immune responses. These properties are being exploited in TLR-based vaccines and therapeutic agents in mammalian species and chickens (St Paul et al., 2013; Steinhagen et al., 2011; Wilson-Welder et al., 2009). In the poultry industry, most vaccinations are achieved either *in ovo* or within 1 week post-hatch. The immune function of chickens is limited in the first 2 weeks post-hatch (Bar-Shira et al., 2003; Mast and Goddeeris, 1999; Reemers et al., 2010b), but adjuvants and the use of potent antigen-delivery systems may counteract age-related defects in immune responses to vaccination.

Multiple studies have shown that TLR-Ls increase vaccine efficacy in chickens. Stimulation of TLRs with TLR-Ls leads to the activation of different transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor 3 (IRF3)), resulting in the production of cytokines and chemokines that enhance proliferation, and increase the capacity of innate immune cells to present antigen. Administering the TLR2 ligand Pam3Cys-SK4 (PCSK) as a vaccine adjuvant significantly enhanced antibody-mediated immune responses by up-regulating antigen-specific IgY (Erhard et al., 2000). Additionally, PCSK up-regulates the Th1 associated cytokines IFN-γ and IL-12, along with the Th2 associated cytokine IL-4 (St Paul et al., 2012). Administration of TLR4 ligands via the respiratory routes or *in ovo* (Barjesteh et al., 2015) increased protective responses to avian respiratory virus infections, through increased type I IFNs (Parvizi et al., 2014) or higher antibody titres (Tseng et al., 2009). Cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (CpG ODN) has been the most widely used TLR-L in chickens to enhance the immunogenicity of vaccines against bacterial (Gomis et al., 2003; Taghavi et al., 2008), protozoal (Dalloul et al., 2005) and viral (Dar et al., 2014; Singh et al., 2015) infections. Like LPS, CpG ODN is a strong inducer of nitric oxide (NO) production and type I and II IFNs

(Barjesteh et al., 2014), but the type and dose of CpG ODN used has an effect on the immune responses (Reemers et al., 2010a; Singh et al., 2015). Synergistic effects of co-stimulation of TLRs on chicken immune responses have been reported using TLR3 and TLR21, leading to enhanced NO production (He et al., 2007), and up-regulation of IFN-γ and IL-10 (He et al., 2012) in PBMC compared to treatment with poly I:C and CpG alone.

In this study, we investigated the synergistic effect of co-stimulation of membrane and endosomal TLRs on the innate immune responses using chicken bone marrow-derived macrophages (BMMs). We examined the innate immune responses to various TLR-Ls in BMMs derived from two different ages: 1-week-old or 4-week-old birds to determine the effect of age on the induction of innate responses. The most potent TLR-Ls, class B CpG (ODN (CpG B), PCSK and synthetic monophosphoryl lipid A (MPLA) were then tested for potential synergistic effects in 4-week-old birds.

2. Materials and methods

2.1. TLR ligands

The synthetic lipoprotein PCSK (EMC microcollections, Germany) for TLR2, MPLA, Di[3-deoxy-D-manno-octulosonyl]-lipid A ammonium salt (KLA; Avanti Polar Lipids, US) for TLR4 (Braun et al., 2017), CpG B (Chrząstek et al., 2014) and CpG C (Invivogen, US) for TLR21 were reconstituted with sterile PBS following manufacturer's instructions. Gardiquimod and Resiquimod (R848) (Chemdea, US) for TLR7 (Braun et al., 2017) were resuspended with DMSO (Sigma-Aldrich, US) following manufacturer's instructions.

2.1 Isolation and culture of chicken BMMs

Commercial Novogen Brown layers were hatched and housed in premises licensed under a UK Home Office Establishment License in full compliance with the Animals (Scientific Procedures) Act 1986 and the Code of Practice for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Requests for animals were approved by the local Animal Welfare and Ethical Review Board and animals were humanely culled in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986.

BMMs from 1-week or 4-week-old birds were cultured as described previously (Kim et al., 2018). Purified cells were seeded in 24-well (Griess test) and 6-well plates (gene expression), at 0.5 x 10^6 cells/well and 2 x 10^6 cells/well respectively, and differentiated in the presence of recombinant chicken CSF1 for 6 days, followed by stimulation with TLR-Ls.

2.2 Stimulation of BMMs with TLR-Ls

To test the optimal concentration, as well as the effect of age on the immune responses, TLR-Ls were used at the following concentrations for 6 and 48 hours (hr): PCSK, MPLA and KLA (0.01, 0.1, 1, 10 μg/mL), Gardiquimod, R848, CpG B and CpG C (0.05, 0.5, 5 μg/mL). Ultra-pure LPS (Kogut et al. 2005) was used as a positive control and a medium control with either PBS or DMSO was used as a control for the TLR-Ls solvents. Combinations of PCSK (0.01 and 0.1 μg/mL), MPLA (0.1 and 1 μg/mL), and CpG B (0.5 and 5 μg/mL) were used in co-stimulation and cell proliferation assays. BMMs were stimulated for 6 hr to measure gene expression and for 48 hr to measure produced nitrite (NO_2^-) in the media by the Griess test (Kim et al., 2009).

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the stimulated BMMs using the RNeasy Mini Spin Column (Qiagen, US) and 1 μg of RNA was reverse transcribed with iScript™ cDNA synthesis kit (Bio-Rad, US), followed by 1:5 dilution for IL-1β, IL-12p40 and IFN-γ and 1: 100 dilution for 28S ribosomal RNA. For IL-10 mRNA levels, undiluted cDNA was used as a template. To measure mRNA levels from the BMMs, 1 μL of diluted cDNA was mixed with 5 μL of SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad) and forward/reverse primer (500 nM final concentration) (Table 1) in nuclease-free water (final volume 10 μL), followed by qRT-PCR reaction described previously (Kim et al., 2009). Target gene expression was normalised against the expression of 28S rRNA, and the relative gene expression was compared to a medium (PBS or DMSO-treated) control sample (Kaiser et al., 2000).

2.4. Stimulation and cell proliferation of splenocytes by TLR-Ls

Splenocytes from 4-week-old birds were purified as described before (Sutton et al., 2015), resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 2% L-glutamine, 1 U/mL penicillin and 1 μg/mL streptomycin (Gibco, US).

Splenocytes were stimulated with two concentrations of TLR2, TLR4 and TLR21 ligands, followed by measuring cell viability and cell proliferation using flow cytometric analysis. Splenocytes were seeded in a 96-well plate at 5.0 x 10^5 cells/well, and an equal volume of the TLR-Ls was added. ConA (5 μg/mL final concentration) was used as a positive control and medium with PBS as a negative control. After 48 hr, the splenocytes were incubated with 2 mM EDTA, and harvested by centrifugation at 300 x *g* for 5 minutes (min) (Dalgaard et al., 2016). The cells were washed twice and resuspended with cold FACS buffer (0.5% BSA and 0.05% Sodium Azide (Sigma-Aldrich) in PBS) containing SYTOX Blue (Invitrogen, US). Cells were analysed by flow cytometry using a BD LSR Fortessa (BD Biosciences) and the

data were analysed with FlowJo (Three Star, USA). Samples were recorded for 10,000 events in the leukocyte gate based on SSC-FSC pattern, followed by gating for single cells. Cell viability was determined by SYTOX Blue staining and the proliferation by forward light scatter (Gaines et al., 1996; MacDonald et al., 1982).

2.5. Statistical analysis

All data were analysed by either the Student's *t*-test or one-way analysis of variance using program R (R Development Core Team, 2015), and significant differences between groups were considered significant by Tukey's honest significant difference test at $P < 0.05$ (confidence level = 95%). Synergistic effect was determined if the NO production and mRNA expression value from the co-stimulatory treatment was significantly higher than the combined value from both treatments with each agonist alone (He et al., 2007).

3. Results and Discussion

In the poultry industry, most vaccinations are achieved either *in ovo* or within 1 week post-hatch. Since the immune function of birds is limited in the first 2 weeks post-hatch (Bar-Shira et al., 2003; Mast and Goddeeris, 1999; Reemers et al., 2010b), potent immune activators are of importance to counteract the age-related defects in immune responses. A potent adjuvant can activate innate immunity through the pattern-recognition receptors, thereby providing the inflammatory context for the rapid recruitment and activation of innate immune cells, which condition the inflamed site for the initiation of adaptive immune responses (Banchereau et al., 2000; Iwasaki and Medzhitov, 2004). Our study examined the effect of age on the innate immune responses to various TLR-Ls in macrophages, and tested the synergistic effect of co-stimulation of membrane and endosomal TLRs.

BMMs were stimulated with various TLR-Ls: PCSK for TLR2, MPLA and KLA for TLR4, Gardiquimod and R848 for TLR7 and CpG B and C for TLR21 (Figure 1). Their ability to activate the BMMs from 1- and 4-week-old chickens was analysed by measuring IL-1β, IL-12p40 and IL-10 for gene expression at 6 hr and NO production at 48 hr.

Nitric oxide is a multi-functional mediator with diverse physiological and pathological roles in host defence and the production of NO is an important aspect of the activation of macrophages. BMMs from 4-week-old birds produced significantly higher levels of NO at 48 hr post-stimulation compared to those from 1-week-old birds (Figure 1A) independent of the TLR-L used. Stimulation of BMMs with TLR-Ls for 6 and 12 hr produced no or very little NO, whereas stimulation of BMMs with TLR-Ls for 72 hr led to cell death based on microscopic observation (data not shown). We measured NO production at 48 hr post-stimulation in our further study. The levels of NO produced by macrophages from 1-week-old birds were less than 10 μM. Low levels of NO production from 1 to 2-week-old chicken PBMC after stimulation with TLR-Ls has been reported previously (He et al., 2007; Gupta et al., 2014; Ramakrishnan et al., 2015). Stimulation of BMMs with PCSK, R848, CpG B, or CpG C led to the highest NO production. In general, an increasing dose led to slightly increased NO production. BMMs from 1-week-old chickens died more rapidly using higher concentrations of TLR-Ls, especially after stimulation with Gardiquimod or R848, based on a microscopic observation.

To investigate the ability of TLR-Ls to act as potential vaccine adjuvants and induce innate responses in macrophages, mRNA levels were measured for IL-1β and IL-10, indicators for pro- and anti-inflammatory cytokines, and IL-12p40 as an indicator for priming Th1 responses. A TLR-L based vaccine adjuvant would preferably induce IL-1β and IL-12p40, but low levels of IL-10 to prevent suppression of the vaccine responses (Coffman, 2010; Darrah et

al., 2010; Stober et al., 2005). All TLR-Ls induced IL-1β mRNA in BMMs, independent of the age of the birds (Figure 1B). BMMs from 4-week-old birds showed significantly higher fold change of IL-1β (fold increase of 3-768) compared to BMMs from 1-week-old birds (fold increase of 1.6-81). The CpG C (0.5 μg/mL) induced highest fold changes of IL-1β (768-fold induction), while the Gardiquimod (0.05 μg/mL) showed the lowest induction of IL-1β (2.9-fold induction) in BMMs from 4-week-old birds. All TLR-Ls induced a dose-dependent response, but the kinetics of the response was dependent on the stimulus. TLR7 ligands induced more IL-1β with increasing concentration, whereas TLR2 and some TLR4 ligands showed an opposite trend. Macrophages are a major source of IL-1β, which is a pivotal pro-inflammatory cytokine for host-defence responses to infection (Gaestel et al., 2009). Additionally, IL-1β functions as an immuno-adjuvant by increasing vaccination efficacy (Deryabin et al., 2014; Dinarello, 2009).

Unlike the induction of IL-1β, stimulation of BMMs from 1-week-old birds did not induce IL-12p40 mRNA. IL-12p40 mRNA levels in BMMs from 4-week-old birds was significantly increased by PCSK (0.01 and 10 μg/mL), LPS, KLA (all concentrations), MPLA (1 and 10 μg/mL), CpG B and CpG C (all concentrations) (Figure 1C). The dose-dependent kinetics of the response differed for each ligand and no consistent pattern was found. TLR7 ligands, Gardiquimod and R848 did not induce IL-12p40 mRNA levels regardless of the age of the bird. IL-12 is a heterodimeric (p35/p40) regulatory cytokine, predominantly produced by B cells, monocytes, macrophages and dendritic cells, that induces IFN-γ production and therefore plays an important role in the development of Th1 immunity (Balu and Kaiser, 2003; Gee et al., 2009).

IL-10 mRNA was not detected in BMMs from 1-week-old birds after stimulation with all tested TLR-Ls (Figure 1D). BMMs from 4-week-old birds had significantly increased IL-

10 mRNA levels after stimulation with MPLS, KLA (all concentrations), R848 (0.05 and 0.5 μg/mL), CpG B and CpG C (all concentrations). The dose-dependent kinetics suggested a trend toward higher induction of IL-10 with increasing dose. Unlike our study, He et al. (2012) reported a significant induction of IL-12p40 and IL-10 mRNA levels in chicken PBMC from 2- or 3-day-old chicks at 4 hr post-stimulation with CpG. These apparent differences may be explained by the use of different CpG motifs and the mixed cell population found in PBMC, compared to BMMs, that can produce IL-12p40 and IL-10, including NK cells and B cells (He et al., 2003; Vollmer et al., 2004). IL-10 is produced by macrophages, dendritic cells, B cells, $CD4^+$ Th2 cells and $CD8^+$ T cells (O'Garra and Vieira, 2007; Rothwell et al., 2004). IL-10 is anti-inflammatory, suppresses pro-inflammatory cytokine secretion, and acts as a master regulator to function as a negative feedback control mechanism to prevent over-expression of both Th1 and Th2 immune response (Couper et al., 2008).

To determine if the combined stimulation of a membrane and endosomal TLR would induce a synergistic effect, BMMs were stimulated with CpG B and either PCSK or MPLA, and NO production, IL-1β, IL-10 and IL-12p40 mRNA expression were examined (Figure 2). Only a combination of low concentration of CpG B (0.05 μg/mL) with low concentration of MPLA (0.1 μg/mL) induced significantly enhanced NO production ($P = 0.04$) compared to the combined NO production of low concentration of CpG B alone and low concentration of MPLA alone (Figure 2A). No synergistic effect was observed with the combination of CpG B and PCSK. Similar to our study, co-stimulation of CpG with PCSK, peptidoglycan, lipoteichoic acid, LPS, flagellin, loxoribine, R837 or single-stranded polyU did not enhance NO production in chicken PBMC, although the PBMC were isolated from birds less than 1 week of age (He et al., 2007). Only a combination of CpG and poly I:C led to the synergistic enhancement of NO production.

Increased mRNA levels of IL-1β in BMMs were observed following stimulation with all tested TLR-L alone and in different combinations. Co-stimulation of BMMs with either low concentration of CpG B (0.5 μg/mL) and high concentration of MPLA (1 μg/mL) (P = 0.03), or high concentration of CpG B (5 μg/mL) and high concentration of PCSK (0.1 μg/mL) (P = 0.04) showed significantly enhanced IL-1β mRNA levels compared to the combined value of each TLR-L alone (Figure 2B). He et al (2007, 2012) reported a synergistic effect of CpG and poly I:C on NO production, as well as mRNA levels of IFN-γ and IL-10 in chicken PBMC, but a synergistic effect of CpG and poly I:C on IL-1β expression in chicken PBMC was only observed at 8 and 24 hr post-stimulation (He et al., 2011).

Similar to IL-1β, all TLR-Ls alone significantly induced mRNA levels of IL-12p40. However, only a combination of low concentration of MPLA (0.1 μg/mL) with either low (0.5 μg/mL) or high (5 μg/mL) concentration of CpG B induced a significant synergistic effect (P = 0.04) on IL-12p40 mRNA levels (Figure 2C). Co-stimulation of chicken PBMC with CpG and poly I:C led to lower IL-12p40 mRNA expression (He et al., 2012). Co-stimulation with CpG B and PCSK also showed down-regulation of IL-12p40 mRNA expression compared to the combined value of CpG B and PCK alone; however, the difference was not significant.

BMMs treated with TLR-L alone induced significant mRNA levels of IL-10, but no synergistic effect of co-stimulation with CpG and either PCSK or MPLA was observed in BMMs (Figure 2D). Co-stimulation of PBMC from birds less than a week old with CpG and poly I:C leads to a significant synergistic effect on IL-10 mRNA levels from 2 hr to 8 hr post-stimulation (He et al., 2012). Inducing the regulatory cytokine IL-10 at early time points may negatively affect innate and adaptive responses.

We also examined co-stimulation with Gardiquimod, a TLR7 ligand, and PCSK or MPLA, but we observed significant cell death during the stimulation, based on microscopic observation.

Finally, we examined the effect of the selected TLR-Ls on lymphocyte proliferation using flow cytometric forward light scatter (Gaines et al., 1996; MacDonald et al., 1982). Incubation of splenocytes with PCSK, MPLA and CpG B did not induce lymphoblasts, whereas ConA induced extensive lympho-blastogenesis (Figure 3). In contrast, stimulation of splenocytes with 5 μg/mL CpG B led to approximately 12% increased cell death compared to splenocytes treated with ConA. Stimulation with 5 μg/mL CpG B has been used previously to stimulated PBMCs and cells from the Harderian gland (He et al., 2007; Chrząstek et al., 2014). We also observed increased death of splenocytes by 0.5 μg/mL of CpG B; however, this effect varied from bird to bird. Similarly, CpG-induced apoptosis has been reported in several species, including fish (Jung and Jung, 2017), avian (Xie et al., 2003), and mammals (Hussein et al., 2016; Krogmann et al., 2016). One speculation is that CpG-induced cell death may provide the danger signal that the host immune system needs in order to respond to the antigen as it would during vaccination. Due to significantly low cell viability following single TLR-L treatment, further studies to validate the synergistic effect of co-stimulation with membrane and endosomal TLRs on splenocytes proliferation were omitted.

In summary, BMMs originating from 4-week-old birds showed better innate immune responses to various TLR-Ls compared to those from 1-week-old birds, implying the need for re-consideration of vaccination in early age (less than 7-day-old) of chicks to maximise the efficiency of vaccine and adjuvant. Although co-stimulation with TLR21 and TLR4 synergistically up-regulated inflammatory-related genes, as well as NO production, incubation of splenocytes with CpG B induced significant cell death without increased cell proliferation.

Conflict of interest

The authors declare that they have no competing interest.

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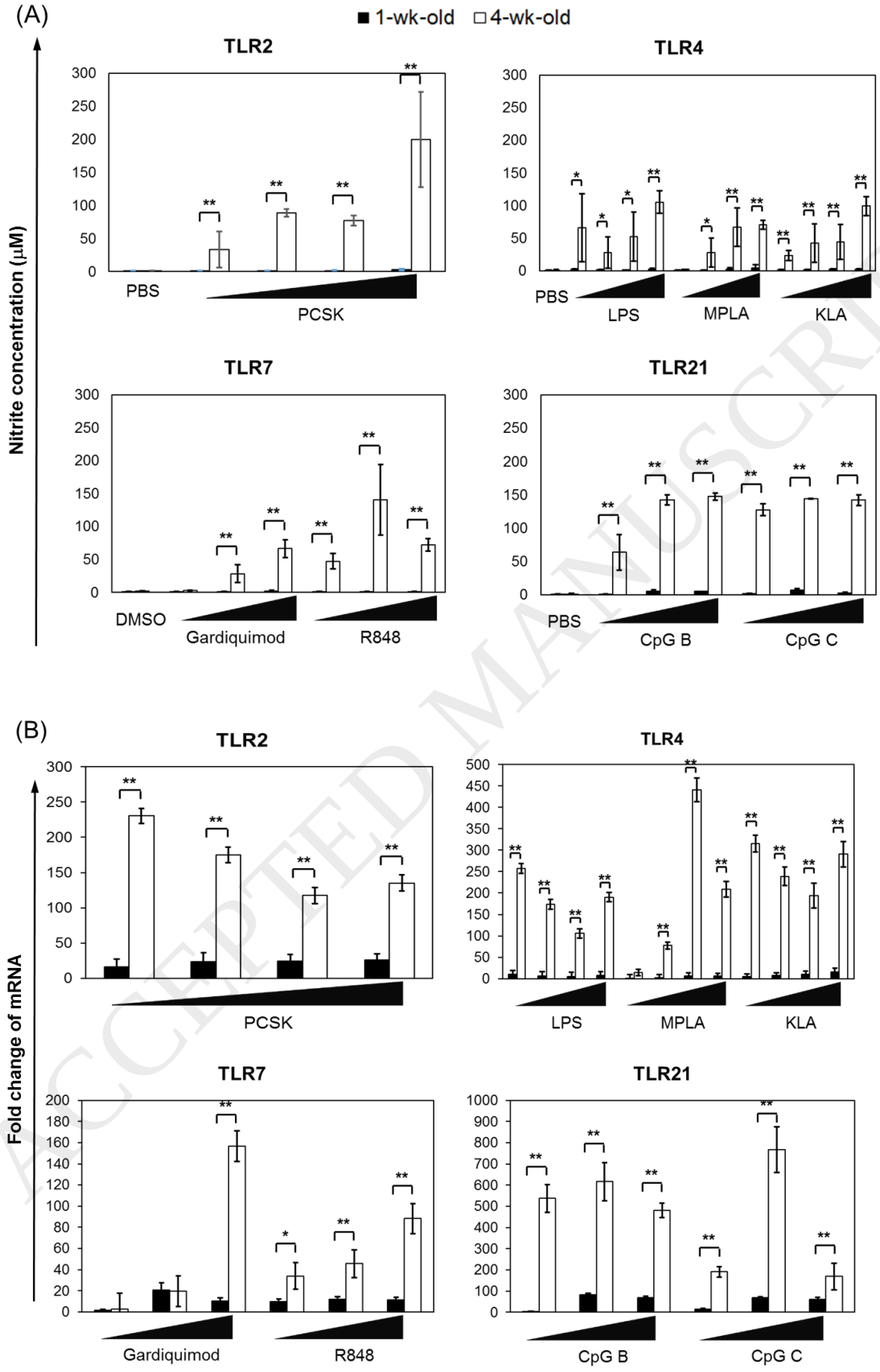
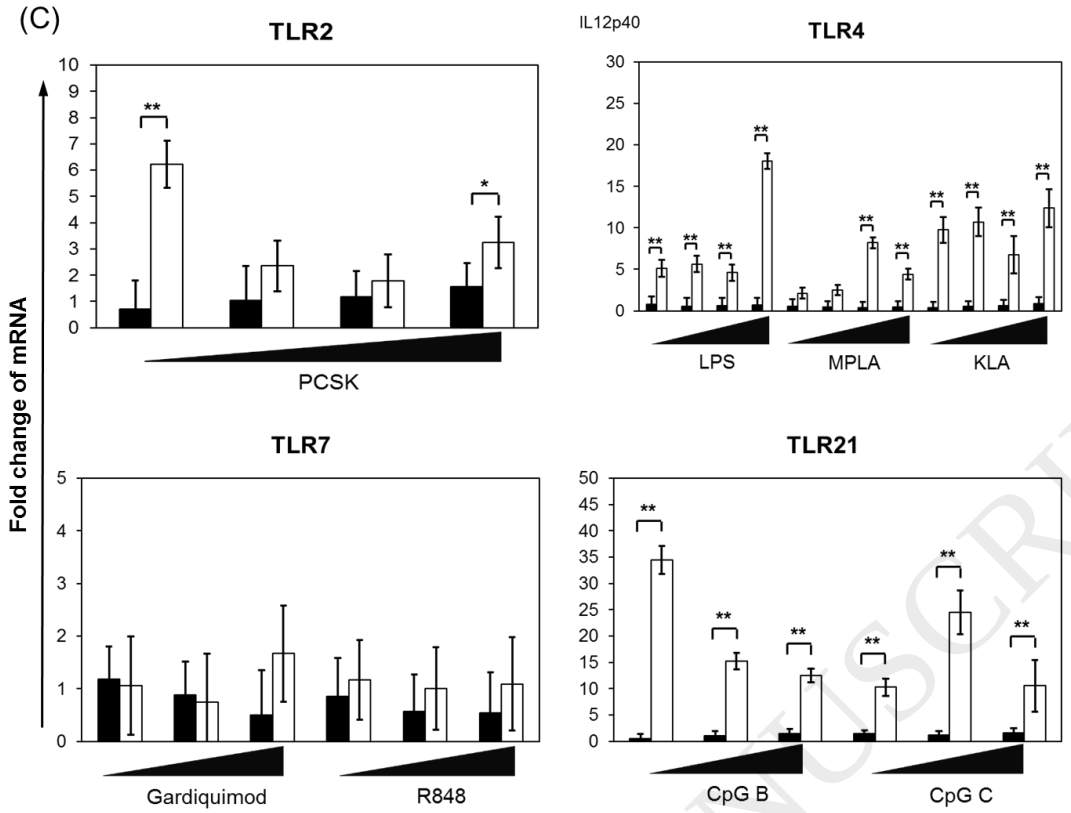
Figure legends

Figure 1. Comparison of innate immune responses of macrophages originated from 1- and 4-week-old birds. Macrophages from 1- or 4-week-old birds were stimulated with various TLR-Ls: PCSK, LPS, MPLA and KLA (0.01, 0.1, 1, 10 μg/mL), Gardiquimod, R848, CpG B and CpG C (0.05, 0.5, 5 μg/mL). Produced NO was measured 48 hr post-stimulation (A). IL-1β (B), IL-12p40 (C) and IL-10 (D) mRNA levels were measured at 6 hr post-stimulation by qRT-PCR. LPS was used as a positive control, and medium with either PBS or DMSO were used as controls for the TLR-Ls solvents. Each bar represents the mean of three individual birds, and error bars are the standard deviation of three individual birds. Asterisks indicate statistically significant differences of NO production and mRNA levels of inflammatory-related cytokines in macrophages from 4-wk-old birds compared to 1-week-old birds. (*P<0.05; **P<0.01).

Figure 1





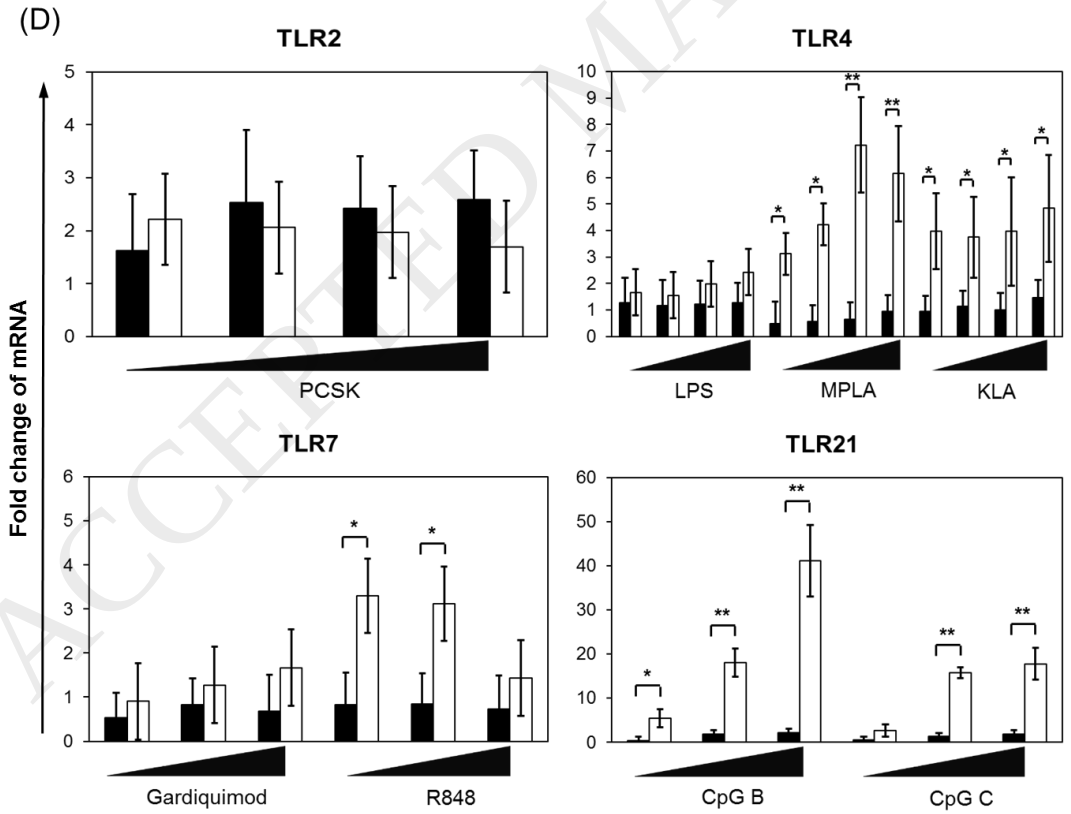


Figure 2. Nitrite production and selected gene expression following co-stimulation with membrane and endosomal TLRs. Macrophages from 4-week-old birds were co-stimulated with the combination of CpG B (0.5 and 5 μg/mL) and PCSK (0.01 and 0.1 μg/mL) or MPLA (0.1 and 1 μg/mL). Produced NO in the media was measured at 48 hr post-stimulation (A) and mRNA levels of IL-1β (B), IL-12p40 (C) and IL-10 (D) were measured at 6 hr post-stimulation. LPS was used as a positive control, and medium with PBS was used as a control for the TLR-Ls solvents. Each bar represents the mean of three individual birds, and error bars are the standard deviation of three individual birds. Asterisk (*) indicates a statistically significant difference between treatments and the control (P<0.05), and Asterisks (**) indicates the statistically significant synergistic effect of a combinatory treatment (P<0.05).

Figure 2

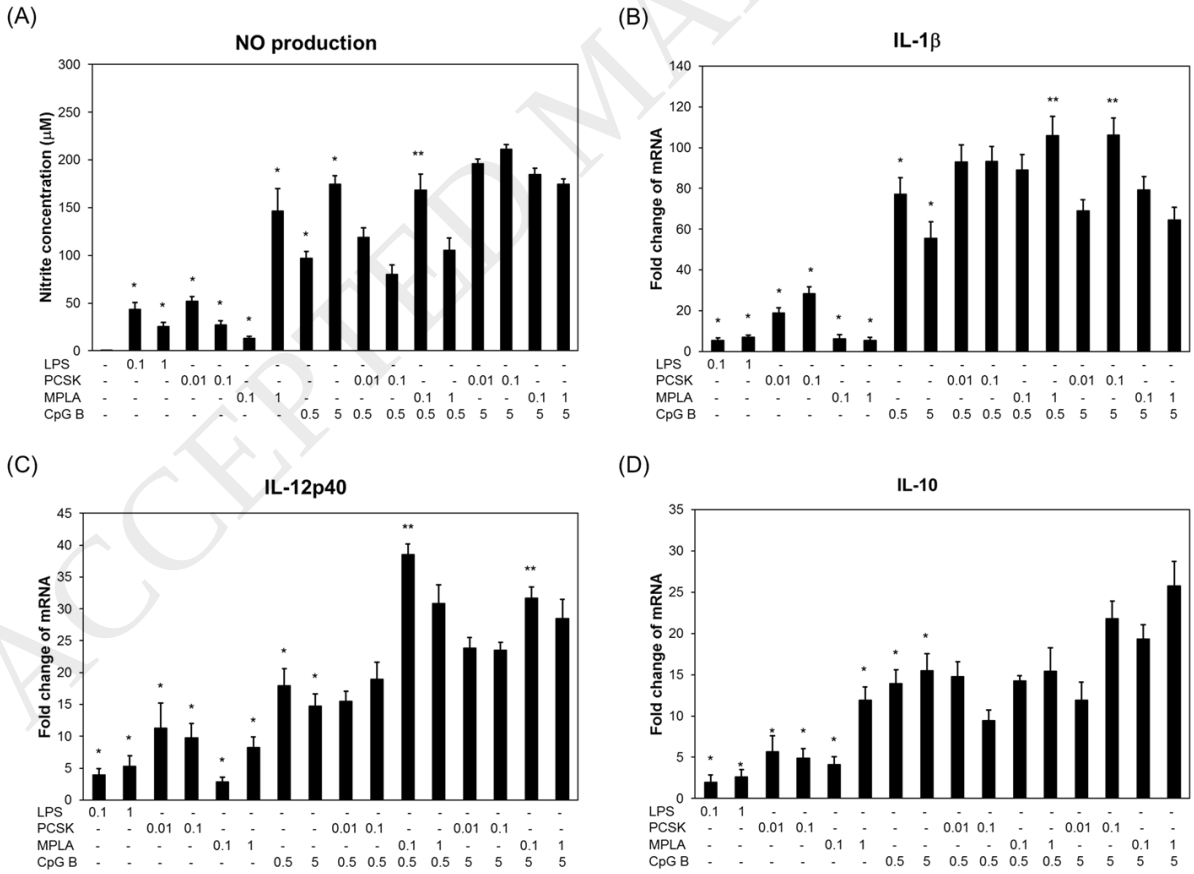


Figure 3. Viability and proliferation of splenocytes after stimulation with TLR-Ls. Splenocytes from 4-week-old birds were stimulated with two different concentrations of PCSK (0.01 and 0.1 μg/mL), MPLA (0.1 and 1 μg/mL) or CpG B (0.5 and 5 μg/mL) for 48 hr. The cells were stained with SYTOX Blue and analysed by flow cytometry to distinguish live and dead cells, and cell proliferation induced by TLR-Ls based on light scatter characteristics. (A) SYTOX Blue positive cells are represented 'Dead' cells, whereas lymphoblasts and non-proliferating cells ('Non-blasts') were gated on light forward scatter characteristics. (B) The bar graph represents a percentage of non-blasts and blasts after stimulation with TLR-Ls. Each bar represents the mean of three individual birds, and error bars are the standard deviation. Asterisk (*) indicates statistically significant differences between non-blasts and blasts (P<0.05), and Asterisks (**) indicate statistically significant differences of live lymphocyte populations (non-blasts + blasts) (P<0.05).

Figure 3

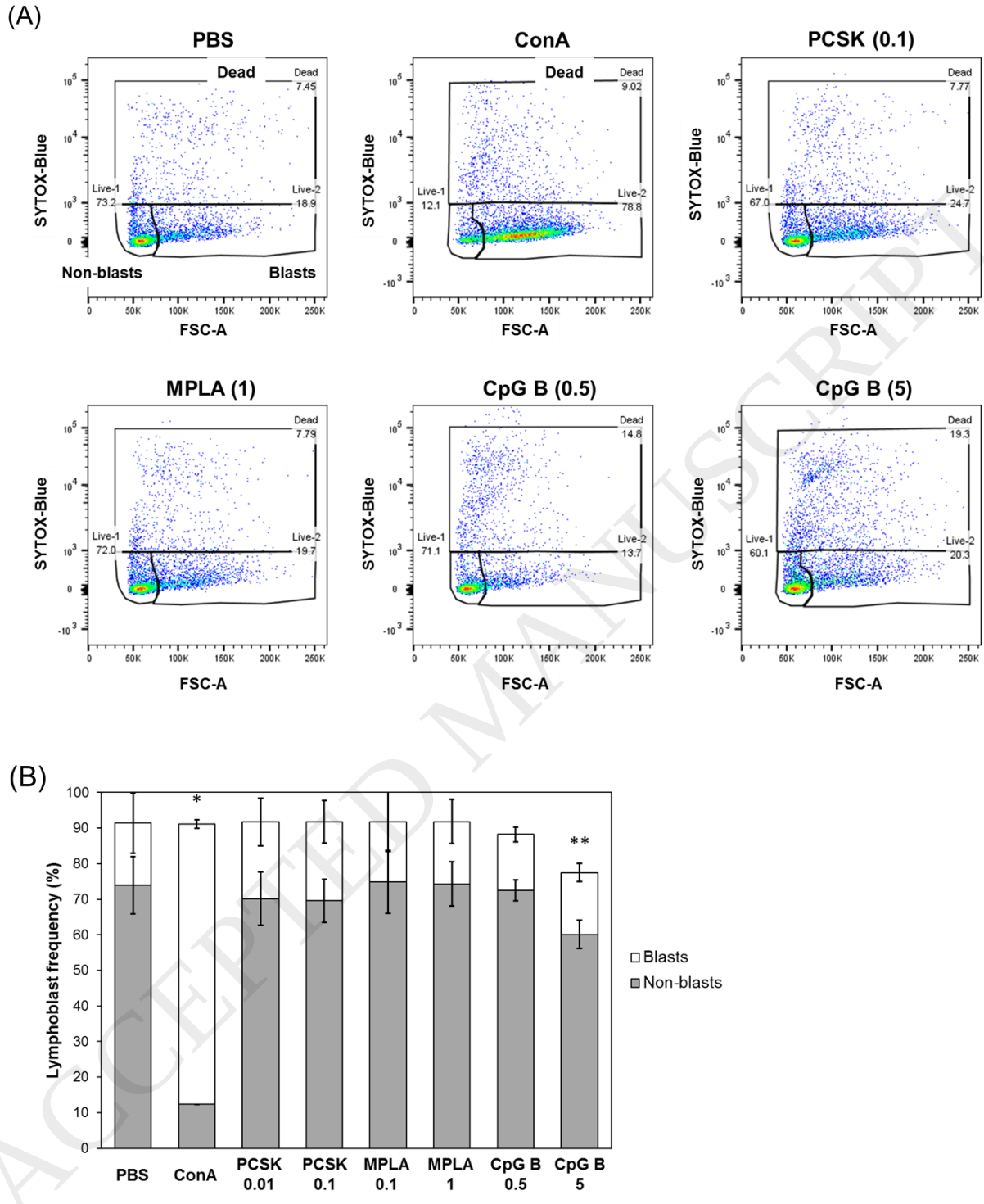


Table 1. Primer sequences for qRT-PCR analyses of cytokine transcripts

Gene	Forward primer	Reverse primer	Reference/ Accession No.
28S	GGCGAAGCCAGAGGAAACT	GACGACCGATTTGCACGTC	(Kaiser et al., 2000)
IL-1β	CAGCAGCCTCAGCGAAGAG	CTGTGGTGTGCTCAGAATCCA	NM_204524
IL-10	GAGTTTAAGGGGACCTTTGGCT	ATGACTGGTGCTGGTCTGCA	NM_001004414
IL-12p40	TGGGCAAATGATACGGTCAA	CAGAGTAGTTCTTTGCCTCACATTTT	NM_213571